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NAD(P)H-flavin oxidoreductase from the bioluminescent bacterium, *Vibrio fischeri* ATCC 7744, is a flavoprotein

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Abstract

The NAD(P)H-flavin oxidoreductase gene from the bioluminescent bacterium, *Vibrio fischeri* ATCC 7744, was expressed in *Escherichia coli*, and the enzyme purified using Cibacron Blue 3G-A affinity column chromatography from crude extracts in a single step. The purified enzyme had a typical flavoprotein absorption spectrum and flavin mononucleotide (FMN) was identified as a prosthetic group, non-covalently bound in a molar ratio of 1:1. The enzyme catalyzed the electron transfer from NADH via $FMNH_2$ to various other electron acceptors. Reduced flavin produced by flavin reductase participated non-enzymatically in the following reactions: H_2O_2-forming NADH oxidase-like, oxygen-insenstive nitroreductase-like, diaphorase (quinone reductase)-like and bacterial luciferase reactions.

Key words: Reduced flavin; Bacterial luciferase; NADH oxidase; Nitroreductase; Diaphorase

1. Introduction

Flavin mononucleotide (FMN or riboflavin phosphate) is one of two coenzymes that is derived from riboflavin (vitamin B_2), the other being flavin adenine dinucleotide (FAD). The 5′ carbon of ribitol of riboflavin is linked to phosphate in FMN, while FAD is an adenylyl derivative of FMN. It is well known that flavins are used widely as cofactors in enzymatic reactions and the functional part of the flavin molecule is the isoalloxazine ring, which serves as a two-electron acceptor/donor [1,2]. The enzymes that utilize a flavin cofactor are called flavoproteins and can react directly with oxygen. Thus, some flavoproteins are classified as oxidases [2,3].

In luminous bacteria, reduced FMN ($FMNH_2$) is an essential component of the luminescence reaction [4] and $FMNH_2$ may be supplied in vivo by NAD(P)H and FMN through the following enzymatic reaction [5,6]:

$$NAD(P)H + H^+ + FMN \rightarrow NAD(P)^+ + FMNH_2$$

The enzyme catalyzing this reaction is known as NAD(P)H : FMN oxidoreductase (EC 1.6.8.1), which is also called FMN reductase or NADH dehydrogenase/oxidase and referred to as flavin reductase in this paper.

It is well characterized that $FMNH_2$, produced enzymatically or non-enzymatically, is rapidly oxidized by molecular oxygen to yield hydrogen peroxide and FMN [7,8], as follows:

$$FMNH_2 + O_2 \rightarrow FMN + H_2O_2$$

However, in luminous bacteria, a free $FMNH_2$ is used for the luminescence reaction [4]. Bacterial luciferase, which has a binding site for $FMNH_2$ [9,10], catalyzes the light producing reaction utilizing $FMNH_2$, molecular oxygen and an aliphatic aldehyde (R-CHO) [11]:

$$FMNH_2 + O_2 + R\text{-}CHO \rightarrow FMN + H_2O + {} + R\text{-}COOH + hv\ (490\ nm)$$

In this reaction, a free $FMNH_2$ binds to luciferase, which is provided by flavin reductase. The flavin reductase of luminous bacteria is quite unique in that it catalyzes a flavin redox reaction with flavins as substrate rather than as tightly bound flavins [8].

Recently, the gene encoding NAD(P)H-flavin reductase was isolated from the bioluminescent bacterium, *Vibrio fischeri* ATCC 7744 (formerly *Achromobacter fischeri* or *Photobacterium fischeri*) [12]. Using the flavin reductase expressed in *E. coli*, it was possible to reconstitute the luminescence reaction in vitro with bacterial luciferase, the same as using native flavin reductase from *V. fischeri* ATCC 7744 [12]. Further, the primary structure deduced from the nucleotide sequence was found to be similar to that of an oxygen-insensitive nitroreductase found in various bacteria [13–15] and to a H_2O_2-forming NADH oxidase from *Thermus thermophilus* HB8 [16], but not to that of flavin reductase (Fre) of *E. coli* [17]. Thus, flavin reductase may be have a dual function in *Vibrio fischeri*. In order to examine this question in more detail, a recombinant form of the enzyme was purified by affinity chromatography. The present paper describes studies on the properties of the enzyme.

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2. Materials and methods

2.1. Materials

The following materials were obtained from commercial sources: β-NADH disodium salt, β-NADPH tetrasodium salt and FMN monosodium salt (Boehringer-Mannheim Co., Indianapolis, IN); FAD, 2,2'-azino-di-(3-ethylbenzthiazoline-6-sulphonic acid) (ABTS) and isopropyl-β-D-thiogalactopyranoside (IPTG; Calbiochem., San Diego, CA); riboflavin, Methylene blue, ferricyanide, 2,6-dichlorophenyl indophenol (DCIP), cytochrome *c* (horse heart), menadione, 5-nitro-3-frualdehyde semicarbazone (nitrofurazone; Sigma Co., St. Louis, MO); 1, 4-benzoquinone and methyl 4-nitrobenzoate (Aldrich Chemical Co., Milwaukee, WI); horseradish peroxidase (Worthington Biochemical Co., Freehold, NJ); Blue Sepharose (Cibacron Blue 3G-A; CL-6B, Pharmacia-LKB, Piscataway, NJ).

2.2. Bacterial strain and plasmid

The *E. coli* strain used for expression was D1210 [18]. The expression plasmid pFR7 used was described previously [12].

2.3. Determination of enzyme activity

2.3.1. NAD(P)H-flavin reductase. The activity was assayed by measuring the initial rate of NAD(P)H oxidation at 340 nm ($\varepsilon = 6.22 \times 10^3$ $M^{-1} \cdot cm^{-1}$) [19] at 22–24°C using an Hitachi (Hitachi, Tokyo, Japan) Model U1100 spectrophotometer. The standard mixture in a final volume of 0.5 ml contained 0.1 mM NAD(P)H and 0.1 mM flavins in 50 mM Na-phosphate buffer, pH 7.0. The reaction was initiated by the addition of NAD(P)H or flavin after preincubation for 1 min at room temperature. The concentrations of FMN, FAD and riboflavin were determined spectrophotometrically using the absorption coefficients of 12.6×10^3 $M^{-1} \cdot cm^{-1}$ (445 nm), 11.3×10^3 $M^{-1} \cdot cm^{-1}$ (450 nm) and 12.5×10^3 $M^{-1} \cdot cm^{-1}$ (445 nm), respectively [20].

2.3.2. NADH-nitrol-quinonel-Methylene blue reductase. The reaction conditions were the same as in the flavin reductase assay, except for the substrate of nitrofurazone, methyl 4-nitrobenzoate, menadione, 1,4-benzoquinone or Methylene blue (0.1 mM each, dissolved in ethanol).

2.3.3. NADH-ferricyanidel-2,6-dichlorophenol indophenol (DCIP)/ cytochrome c reductase. The assay mixture contained potassium ferricyanide (0.1 mM), DCIP (0.1 mM) or cytochrome *c* (2.5 mg), and enzyme in 0.49 ml of 50 mM Na-phosphate buffer, pH 7.0, and was preincubated for 1 min at room temperature. The reaction was initiated by adding 5 μl of 10 mM NADH after 3 min, followed by 5 μl of 10 mM FMN. For the calculation, the absorbances of ferricyanide reduction at 410 nm ($\varepsilon = 1 \times 10^3$ $M^{-1} \cdot cm^{-1}$) [21], DCIP at 600 nm ($\varepsilon = 20.6 \times 10^3$ $M^{-1} \cdot cm^{-1}$) [22] and cytochrome *c* at 550 nm ($\varepsilon = 18.5 \times 10^3$ $M^{-1} \cdot cm^{-1}$) [23] were used.

2.4. Determination of hydrogen peroxide formation in the flavin reductase reaction

After the NAD(P)H-flavin reductase reaction had proceeded for 1 min, the formation of hydrogen peroxide was estimated by adding 20 mM ABTS (5 μl) (as a dye and potent inhibitor of flavin reductase) and 20 U horseradish peroxidase (3,100 U/mg protein) to 0.49 ml of the assay mixture [24]. The amount of hydrogen peroxide was determined by using an absorbance at 414 nm ($\varepsilon = 36 \times 10^3$ $M^{-1} \cdot cm^{-1}$) [24] and by comparing the results with known amounts of hydrogen peroxide (1.32–6.60 nmol).

2.5. Protein analyses

Protein concentration was determined using the dye-binding method of Bradford [25]. A commercially available kit (Bio-Rad, Richmond, CA) was used with bovine serum albumin serving as a standard. SDS-PAGE was carried out under reducing conditions using a 12% separation gel, as described by Laemmli [26].

2.6. Expression and affinity purification of recombinant flavin reductase from E. coli

For over-expression of the protein, *E. coli* D1210 cells transformed with the expression plasmid pFR7 [12] were grown at 37°C in 50 ml Luria-Bertani broth with 5 μl of added Antiform A (Sigma) in a 500 ml Erlenmyer flask accompanied by vigorous shaking in a rotary incubator (300 rpm/min). When the absorbance (600 nm) of the medium reached 0.3, IPTG was added to a final concentration of 0.2 mM and the incubation continued for another 4 h. The cells were harvested by centrifugation and suspended in 30 ml of 30 mM Tris-HCl, pH 7.5, 10 mM EDTA. The cells were disrupted by sonication (15 × 20 s, in an ice bath) using a Branson (Danbury, CT) Model 250 Sonifier. After centrifugation at 12,000 × *g* for 20 min at 4°C in a Beckman Model J2–21B refrigerated centrifuge, the supernatant (28 ml) was stored at 4°C until used. The protein was purified by affinity chromatography at room temperature. The supernatant (25 ml) was introduced directly into a Blue-Sepharose CL-6B column (10 × 45 mm) previously equilibrated with 30 mM Tris-HCl, pH 7.5, 10 mM EDTA at a flow rate of 25 ml per h. The column was washed with the same buffer until the absorbance (280 nm) of the eluent fell below 0.005. The protein was then eluted from the gel with 15 ml of 1 mM NADH.

2.7. Preparation of apo-flavin reductase (apoenzyme) for reductase assays

Using the purified flavin reductase (0.55 mg), the apoenzyme was isolated by reverse-phase HPLC using a 4.6 × 100 mm, 5 mm particle size, 5C4 column (Wako Chemicals, Osaka, Japan) and a linear gradient of 0–80% acetonitrile in 0.1% trifluoroacetic acid run at a flow rate of 0.5 ml per min for 80 min. The apoenzyme eluted as a single peak at a concentration of 18% acetonitrile.

2.8. N-Terminal amino acid sequencing

N-Terminal amino acid sequence analysis was carried out on the peak HPLC fraction using an Applied Biosystem (Foster City, CA) Model 470A gas-phase sequencer fitted with an on-line PTH analytical system.

2.9. Absorption spectra

Absorption spectra of the purified enzyme were determined at room temperature with a Beckman Model DU-50 recording spectrophotometer using a quartz cell (10 mm light path). The purified enzyme (1.37 mg/ml) was dialyzed against 50 mM Na-phosphate buffer, pH 7.0, prior to absorbance measurement. Spectral scans of the protein solution was performed under anaerobic conditions before and after reduction with NADH (1 mM final concentration) and on re-oxidation of the enzyme by shaking the cuvette in air. For obtaining anaerobic conditions, argon gas (99.9%) was bubbled into the cuvette for 5 min.

2.10. Identification of the prosthetic group

The purified enzyme (0.28 mg), dissolved in 0.1 ml of 50 mM Na-phosphate buffer, pH 7.0, was added to 0.9 ml of methanol (HPLC grade) and the solution was heated at 95°C for 15 min in a heating block. After cooling on ice, centrifugation at 12,000 × *g* for 5 min at room temperature yielded a yellow supernatant and the absorption spectrum was measured with a Beckman DU-50 spectrophotometer. A separate aliquot of the supernatant was concentrated in a Speed-Vac concentrator for 3 h in a dark room. The resulting yellow supernatant (2 μl) was also analyzed by thin-layer chromatography employing a silica gel 60 F-254 plate (2 mm thickness; Merck AG, Darmstadt, Germany) and *n*-butanol/glacial acetic acid/water (4:1:5) as the solvent phase [27]. Methanol solutions (2 μl) of riboflavin, FMN and FAD (0.2 mM each) were used as standards and the migration of the compounds was monitored by the characteristic fluorescence in UV light (312 nm). R_f values of riboflavin, FMN and FAD were 0.49, 0.22 and 0.13, respectively.

3. Results and discussion

The Blue Sepharose CL-6B gel containing the dye, Cibacron Blue 3G-A, which binds enzymes requiring adenylyl-type cofactors [28], bound the expressed flavin reductase and was effective as a single step purification of the enzyme. The adsorbed flavin reductase was eluted by 1 mM NADH, but not by 1 mM NAD. SDS-PAGE analysis showed the enzyme to be >95% homogeneous (Fig. 1). The recovery of activity was 92% from cell extracts, with 7.8 mg of protein obtainable from 300 ml

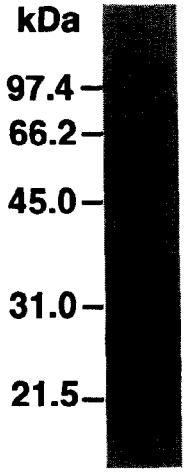

Fig. 1. SDS-PAGE analysis of purified recombinant flavin reductase by Blue-Sepharose affinity chromatography. Purified flavin reductase (98 μg protein) was applied to the gel. Molecular weight markers (Bio-Rad): phosphorylase *b* (97,400), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000) and soybean trypsin inhibitor (21,500).

cultured cells. The clear color of the peak fraction eluted by NADH changed to a yellow color in 10 min. Using SDS-PAGE, the yellow compound dissociated from the protein and migrated to the gel bottom. Also, on reverse-phase HPLC, a yellow compound was eluted in the void fraction. Thus, the purified enzyme contained a non-covalently bound prosthetic compound. Recombinant flavin reductase showed good activity (80–100% relative activity) between pH values of 6–8. The eluted enzyme was quite stable and no significant loss in enzyme activity was observed during 12 months storage at 4°C. Heat stability of the enzyme was good; on incubation at 50°C for 20 min, the enzyme still showed full activity. The N-terminal amino acid sequence analysis of the purified protein showed the sequence to be THPIIHDLENRYTSKKYD, beginning with the second position in the N-terminal sequence, as deduced from the gene encoding the flavin reductase [12]. The N-terminal methionine may have been cleaved by methionine aminopeptidase in the *E. coli* cells [29]. Thus, the apparent molecular size of recombinant flavin reductase as determined by SDS-PAGE is 26 kDa (Fig. 1) and the calculated molecular weight is 24,450 Da based on the presence of 217 amino acid residues.

The absorption spectrum of purified flavin reductase had peaks at 375 and 455 nm and shoulders at 360 and 475 nm (Fig. 2, solid line). The peak at 455 nm disappeared when 1 mM NADH was added under anaerobic conditions (Fig. 2, dashed line) and reappeared on oxygenation. The yellow color of the protein was also bleached by the addition of excess sodium hydrosulfite. These spectral properties are typical of a flavin-containing flavoprotein [1,2]. When the purified flavin reductase was denatured in hot methanol, a yellow compound was released, indicating a non-covalent association with the protein. The absorption spectrum of the methanol solution showed peaks at 270, 360 and 445 nm (data not shown), suggesting the presence of an oxidized flavin compound. Thin-layer chromatography of the methanol extract gave a single fluorescent spot with a R_f of 0.22, identical to that of authentic FMN. Thus, the prosthetic group of recombinant flavin reductase is presumably FMN. Based on an extinction coefficient of 12.6×10^3 $M^{-1} \cdot cm^{-1}$ for FMN at 445 nm [20], on an absorbance reading of the methanol extract of the purified protein (0.28 mg protein/ml methanol; $A_{445} = 0.120$), and on the molecular weight being 24,450 Da, 9.5 nmol of FMN was calculated to be associated with 11.4 nmol of purified enzyme (0.83 mol of FMN bound per mol of protein). Thus, the estimated ratio of FMN in flavin reductase would be 1 mol of FMN per mol of protein.

The FMN-containing purified recombinant flavin reductase, in the absence of exogenous flavins, showed a lower reduced activity in the oxidation reaction of NADH to NAD^+, but the addition FMN, FAD or riboflavin to the reaction mixture caused a marked stimulation of the oxidation of NADH (Table 1). On using NADPH, the reductase activity was found to be three times lower than in the presence of NADH, indicating a preference for NADH in the reaction. The apoenzyme lacking FMN also catalyzed the reduction of FMN, FAD and riboflavin. The order of addition of flavins and NAD(P)H did not affect the final activity. These results suggest that FMN, non-covalently bound to apoenzyme, is reduced to $FMNH_2$, which then dissociates from the enzyme and is immediately replaced by another FMN, FAD or riboflavin, repeating the oxidation cycle. This may explain the reason for flavin reductase's broad substrate specificity.

As shown in Table 2, the major product of the flavin reductase reaction under aerobic conditions was hydro-

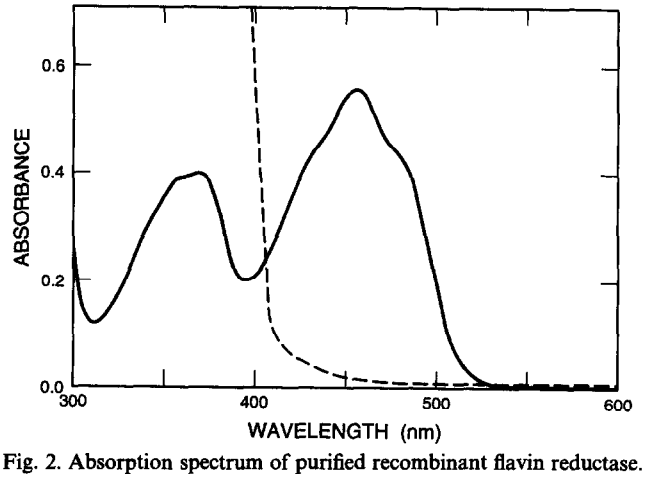

Fig. 2. Absorption spectrum of purified recombinant flavin reductase. The spectrum was measured as described in section 2. The dashed line shows the spectrum in the presence of 1 mM NADH under anaerobic conditions.

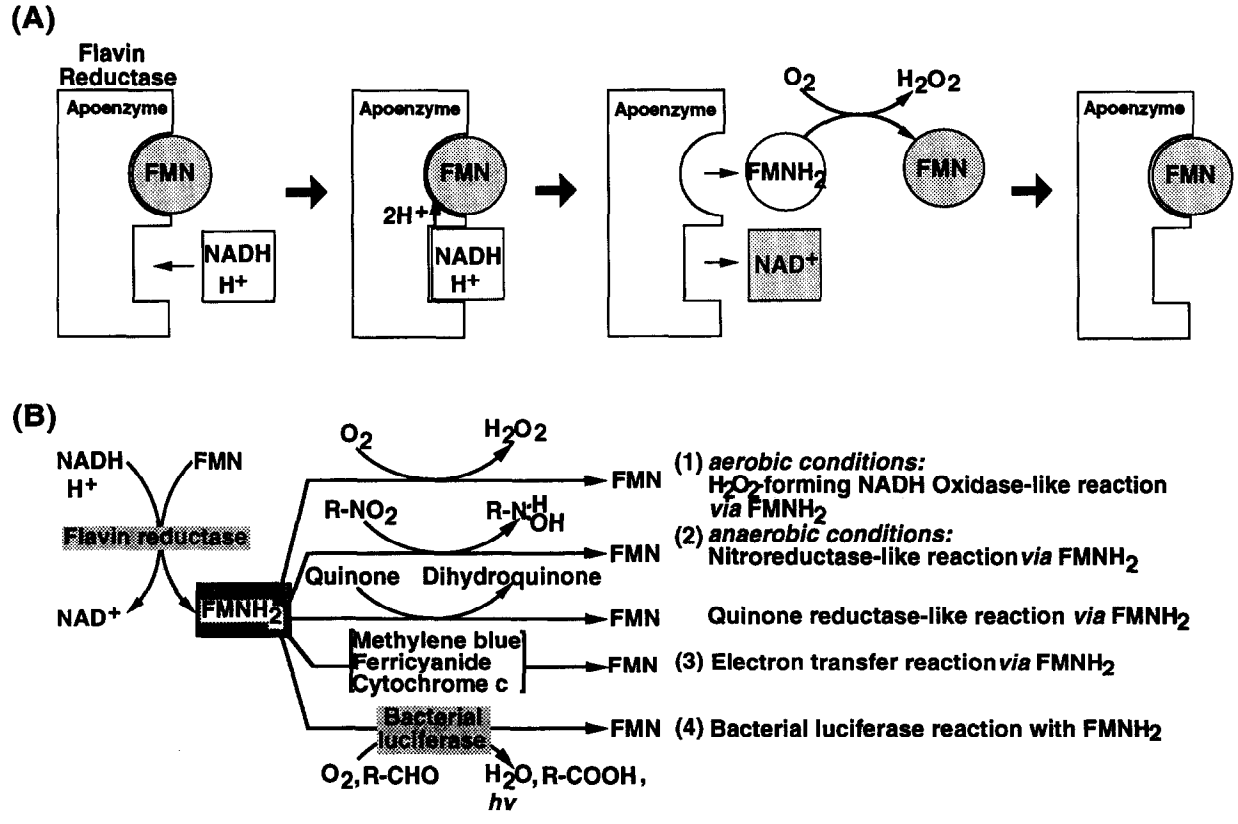

Fig. 3. Schematic representation of the flavin reductase reaction. (A) Reaction scheme of flavin reductase under aerobic conditions. (B) $FMNH_2$-mediated reactions of flavin reductase.

gen peroxide. Either in the presence of FAD and riboflavin, or in the absence of exogenous flavin, hydrogen peroxide was also detected in the reaction mixture. Presumably, free, reduced flavin molecules, released from flavin reductase, reacted non-enzymatically with oxygen dissolved in water to produce the observed hydrogen peroxide and oxidized flavin [7].

As shown in Table 3, Methylene blue and potassium ferricyanide accepted electrons from NADH without addition of exogenous FMN to the enzyme mixture. Efficient transfer of electrons occurred from NADH to cytochrome *c* on addition of FMN to the enzyme mixture. The addition of exogenous FMN to the apoenzyme lacking FMN caused ferricyanide- and cytochrome *c*-reductase activity to be restored effectively. Further, in the absence of exogenous FMN, the enzyme reduced nitroaromatic compounds such as nitrofurazone and methyl 4-nitrobenzoate, and also reduced quinone compounds such as menadione (vitamin K_3) and 1,4-benzoquinone. In contrast, apoenzyme lacking FMN lost over 98% of its capability in transferring electrons from NADH to other electron acceptors. These results suggest that electron transfer from NADH to other electron acceptors is mediated through $FMNH_2$ generated by the flavin reductase.

As shown schematically in Fig. 3A, *V. fischeri* flavin reductase is a flavoprotein containing 1 mol of FMN as a prosthetic group per mol of enzyme. When NADH binds to flavin reductase, two hydrogens (electrons) are transferred by an enzymatic reaction to FMN to yield $FMNH_2$, which causes $FMNH_2$ to be immediately dissociated from the enzyme. Under aerobic conditions, $FMNH_2$ is rapidly oxidized non-enzymatically by molecular oxygen to yield hydrogen peroxide and FMN, upon which the oxidized FMN binds to apoenzyme. The stim-

Table 1
Stimulation of flavin reductase activity on the addition of flavins

Conditions	Enzyme activity (μmol/min/mg protein)
Enzyme	0.0
Enzyme + FMN	0.0
Enzyme + NADH	1.0
Enzyme + NADH + FMN	73.2
Enzyme + NADH + FAD	44.9
Enzyme + NADH + riboflavin	67.6

Enzyme activity was determined using NADH-flavin reductase as described in section 2 at a concentration of 0.1 mM NADH and with or without 0.1 mM flavin. The concentration of purified flavin reductase was 0.4 μg.

ulation of enzyme activity on addition of various flavins (Table 1) and the broad flavin specificities possessed by the enzyme indicate that the flavin recycles in the reaction, being attached in the oxidized form and dissociated in the reduced form.

As described in a previous paper [12], the identity and similarity of the amino acid sequence between flavin reductase and the H_2O_2-forming NADH oxidase of *T. thermophilus* HB8 [16] are 21% and 39%, respectively, and between flavin reductase and an oxygen-insenstive nitroreductase of *Enterobactor cloacae* [14] are 32% and 56%, respectively. The similarity in the primary structures is presumably related to the catalytic function of these enzymes. With flavin reductase, the enzyme catalyzes the formation of hydrogen peroxide and the reduction of nitroaromaic compounds (Tables 2 and 3). In these reactions, the formation of free $FMNH_2$ by flavin reductase appears to be a key step, leading to a free $FMNH_2$ mediated-chemical reaction (Fig. 3B). Under aerobic conditions, $FMNH_2$ (or reduced flavins) is converted to hydrogen peroxide and FMN (or oxidized flavins), as occurs in the case of the H_2O_2-forming NADH oxidase reaction [16]. Under anaerobic conditions, an oxygen-insenstive nitroreductase-like reaction is observed, which is not dependent on oxygen radicals and/or anions as electron donors, and which may be competitive with molecular oxygen. An oxygen-insenstive, FMN-containing nitroreductase catalyzes an obligatory two-electron reduction of nitrocompounds to nitrosoamines, hydroxyamines and amines [30]. A similar chemical reaction in organic solvent has been reported by Gibian et al. [31–33]. The chemically reduced flavins (dihydroflavins) can reduce nitrobenzene derivatives to their hydroxyamine, and also converted azobenzene to hydroazobenzene under anaerobic conditions [32]. Quinones, including 1,4-benzoquinone and 9,10-anthraquinone, are reduced to hydroquinones by reduced flavins [31,33]. The reductase catalyzed electron transfer to menadione and 1,4-benzoquinone presumably follows the same pathway (Table 3, Fig. 3B). Further, non-enzymatic reduction of cytochrome *c*, methemoglobin, menadione and ferricyanide by $FMNH_2$ under anaerobic conditions has also been reported by Cormier et al. [34]. These results suggest that various compounds can be reduced non-enzymatically by reduced flavins produced by flavin reductase.

Table 2
Formation of hydrogen peroxide in the flavin reductase reaction under aerobic conditions

Conditions	H_2O_2 formation (μmol/min/mg protein)
Enzyme[a]	82.5
Heat denatured enzyme[b]	>0.1
Apoenzyme[c]	>0.1

[a] NADH-FMN reductase activity was 76.3 μmol/min/mg protein.
[b] Heated at 95°C, 20 min.
[c] NADH-FMN reductase activity was 17.0 μmol/min/mg protein.

Table 3
Various electron acceptors of flavin reductase

Addition to:	Enzyme activity (μmol/min/mg protein)	
	enzyme	apoenzyme
None[a]	0.9	0.0
+ FMN[a]	72.5	18.0
+ Nitrofurazone[b]	31.1	0.0
+ Methyl 4-nitrobenzoate[b]	57.2	0.0
+ Menadione[b]	66.2	0.0
+ 1,4-Benzoquinone[b]	64.3	0.2
+ Methylene blue[b]	52.3	0.2
+ Ferricyanide[c]	70.0	0.2
+ Ferricyanide + FMN[c]	100.0	86.8
+ Cytochrome c[d]	1.0	0.2
+ Cytochrome c + FMN[d]	56.8	51.0
+ DCIP[e]	3.4	0.0
+ DCIP + FMN[e]	4.9	0.2

The reaction systems consisted of: [a] NADH-FMN reductase, [b] NADH-nitro/-quinone/-Methylene blue reductase, [c] NADH-ferricyanide reductase, [d] NADH-cytochrome *c* reductase, [e] NADH-DCIP reductase. Each assay mixture contained flavin reductase (0.4 μg) or apoenzyme (1.98 μg) and NADH at a final concentration of 0.1 mM; final FMN concentration was 0.1 mM.

In the bacterial bioluminescence reaction, a free $FMNH_2$ produced by flavin reductase binds to the luciferase molecule [9,10]. The inhibition of luminescence previously reported in extracts of *V. fischeri* by methemoglobin [34], cytochrome *c* [35], menadione [36,37] and ferricyanide [37] may also be explained by competition of free $FMNH_2$ by bacterial luciferase and other compounds in these reactions (Fig. 3B).

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